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(71) Applicant *(for all designated States except US)*: BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA [US/US]; 600 Parrington Oval, Norman, OK 73109 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CUMMINGS, Richard, D. [US/US]; 5215 Santa Fe, Edmond, OK 73034 (US). MOORE, Kevin, L. [US/US]; 612 N.W. 42nd Street, Oklahoma City, OK 73118 (US). MCEVER, Rodger, P. [US/US]; 1716 Guilford Lane, Oklahoma City, OK 73120 (US).

(74) Agents: PABST, Patrea, L. et al.; Kilpatrick & Cody, 1100 Peachtree Street, Suite 2800, Atlanta, GA 30309-4530 (US).

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(54) Title: GLYCOPROTEIN LIGAND FOR P-SELECTIN AND METHODS OF USE THEREOF

(57) Abstract

P-selectin has been demonstrated to bind primarily to a single glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. This molecule was characterized and distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass. The purified ligand, or fragments thereof, including both the carbohydrate and protein components, or antibodies to the ligand, or fragments or components thereof, can be used as inhibitors of binding of P-selectin to cells.

GLYCOPROTEIN LIGAND FOR P-SELECTIN AND METHODS OF USE THEREOF

Background of the Invention

The United States government has rights in this invention as a result of National Institutes of Health grants HL 34363 (R.P. McEver) and HL 45510 (R.P. McEver and K.L. Moore), CA 38701 (A. Varki), IT4 RR 05351 (R.D. Cummings), and GM 45914 (D.F. Smith).

The selectins are three structurally related membrane glycoproteins that participate in leukocyte adhesion to vascular endothelium and platelets, as reviewed by McEver in *Thromb. Haemostas., 66*: 80-87 (1991) and in *Curr. Opin. Cell Biol., 4*; 840-849 (1992). P-selectin (CD62), previously known as GMP-140 or PADGEM protein, is a receptor for neutrophils, monocytes and subsets of lymphocytes that is rapidly translocated from secretory granule membranes to the plasma membrane of activated platelets, as reported by Hamburger and McEver, *Blood 75*: 550-554 (1990); Larsen et al., *Cell 59*: 305-312 (1989) and endothelial cells, as reported by Geng et al., *Nature, 343*: 757-760 (1990); Lorant et al., *J. Cell Biol., 115*: 223-234 (1991).

E-selectin (ELAM-1) is a cytokine-inducible endothelial cell receptor for neutrophils, as reported by Bevilacqua et al., *Proc. Natl. Acad. Sci. USA, 84*: 9238-9242 (1987), monocytes, as reported by Hession et al., *Proc. Natl. Acad. Sci. USA, 87*: 1673-1677 (1990), and memory T cells, as reported by Picker et al., *Nature (London), 349*: 796-799 (1991); Shimizu et al., *Nature (London), 349*: 799-802 (1991). L-selectin (LAM-1, LECAM-1), a protein expressed on myeloid cells and most lymphocytes, participates in neutrophil extravasation into inflammatory sites and homing of

been estimated at 10,000-20,000 per cell (Moore et al., 1991; Skinner et al., 1991), suggesting that these sites constitute a small component of the total cell surface protein. The protein portion of this ligand(s) may be crucial for binding by presenting the glycan in an optimal configuration, clustering glycans to enhance avidity, favoring the formation of specific oligosaccharide structures by cellular glycosyltransferases or modifying enzymes, and/or stabilizing the lectin-carbohydrate interaction through protein-protein interactions with P-selectin.

The potential importance of protein components in enhancing ligand affinity is supported by studies of CHO cells transfected with a specific fucosyltransferase (Zhou et al., (1991)). These cells express higher amounts of the sialyl Le^x antigen than do HL-60 cells and have protease-sensitive binding sites for P-selectin. However, the interaction of P-selectin with these sites is of much lower apparent affinity than with those on myeloid cells, and adhesion of transfected CHO cells to immobilized P-selectin is weaker than that of neutrophils and HL-60 cells (Zhou et al., (1991)). These observations suggest that myeloid cells express one or more membrane glycoproteins not found on CHO cells that enhance the lectin-mediated interaction with P-selectin. Alternatively, myeloid cells may express a glycosyltransferase or modifying enzyme not present in CHO cells.

It is therefore an object of the present invention to identify and characterize a specific glycoprotein ligand for P-selectin (CD62).

It is a further object of the present invention to provide methods and compositions derived from the characterization of a specific glycoprotein

demonstrated to bind to sialylated, fucosylated lactosaminoglycans (including the tetrasaccharide sialyl Lewis x (sLe^x)) on both myeloid and nonmyeloid cells.

The ability of proteases to abolish P-selectin binding to neutrophils indicated that high affinity binding of P-selectin to myeloid cells occurred through interactions with cell surface glycoprotein(s) rather than with glycolipids. As also described in WO 92/01718 P-selectin bound preferentially to a glycoprotein in human neutrophil extracts of Mr 120,000 daltons (D), as analyzed by SDS-PAGE under reducing conditions. The glycoprotein was partially purified on a P-selectin affinity column. It appeared to be heavily glycosylated because it stained poorly with silver and Coomassie blue. It appeared to be heavily sialylated because it bound to a wheat germ agglutinin affinity column. Treatment of the glycoprotein ligand with low doses of sialidase slowed its mobility on SDS gels, a pattern consistent with partial desialylation of heavily O-glycosylated proteins. Binding of P-selectin to the glycoprotein ligand was Ca^{2+}-dependent, blocked by monoclonal antibodies to P-selectin that also block P-selectin binding to leukocytes, and abolished by extensive treatment of the ligand with sialidase.

The preferential binding of P-selectin to the 120,000 D glycoprotein ligand in myeloid cell extracts suggested that it contained special structural features that are recognized with high affinity by P-selectin. Such structures might not be present on every protein or lipid characterized by sialylated, fucosylated structures such as sLe^x. It has now been further demonstrated that the adhesion of myeloid cells to immobilized P-selectin

Behring Corp. (La Jolla, CA), Micro BCA protein assay kits and Lubrol PX (Surfact Amp PX) were purchased from Pierce Chemical Company (Rockford, IL.). Enzymobeads™, Tween-20™, Affigel™-15, and high molecular weight protein standards were from Bio Rad Laboratories (Hercules, CA). Endo-ß-galactosidase (150 U/mg, EC 3.2.1.103) from *Bacteroides fragills*, 4-methyl-umbelliferyl α-*N*-acetylneuraminic acid, and 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid (Neu2en5Ac) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Peptide:*N* glycosidase F (PNGaseF) from *Flavobacterium meningosepticum* (EC 3.2.2.18, N-glycanase) and endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (EC 3.2.1.97, O-glycanase™) were purchased from Genzyme (Cambridge, MA). HBSS was obtained from Gibco Laboratories (Grand Island, NY). Vecta-Stain ABC kits were purchased from Vector Laboratories Inc. (Burlingame, CA). Phycoerythrin-streptavidin was obtained from Becton Dickinson & Co. (San Jose, CA) and phycoerythrin-conjugated anti-mouse IgG_1 was from Caltag (South San Francisco, CA). Rabbit anti-mouse IgG was purchased from Organon Teknika (Durham, NC) and protein A-Sepharose CL4B was from Pharmacia Fine Chemicals (Piscataway, NJ). [6-^{3}H]glucosamine was obtained from Dupont/New England Nuclear (Boston, MA). All other chemicals were of the highest grade available.

Antibodies and Proteins

The anti-P-selectin murine MAbs S12 and G1, and goat anti-human P-selectin IgG were prepared and characterized as described by McEver and Martin, *J. Biol. Chem.*, *259*: 9799-9804 (1984); Geng et al. (1990); Lorant et al. (1991). Rabbit polyclonal antisera and murine MAbs to human lamp-1 (CD3), described by Carlsson et al., *J. Biol. Chem.*, *263*:

Contaminating erythrocytes were lysed by resuspending the pellets with 5 mM EDTA, pH 7.5, in H_2O for 20 s. An equal volume of 1.8% NaCl, 5 mM EDTA, pH 7.5, was then added to restore isotonicity. The cells were centrifuged at 500 x *g* for 5 min and resuspended in ice-cold HBSS containing 5 mM EDTA and 10 mM MOPS, pH 7.5. Diisopropylfluorophosphate was then added to a final concentration of 2 mM and the cell suspension incubated for 10 min on ice. The cells were centrifuged at 500 g for 5 min at 4°C and resuspended in ice-cold 100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.3 (relaxation buffer). To this suspension the following protease inhibitors were added at the indicated final concentrations: 2 mM diisopropylfluorophosphate, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine. The cell suspension was pressurized with N_2 at 350 psi in a cell disruption bomb (model 4635; Parr Instrument Company, Moline, IL.) for 40 min at 4°C with constant stirring as described by Borregaard et al., *J. Cell Biol.*, *97*: 52-61 (1983). The cavitate was collected into EGTA (2 mM final concentration) and nuclei and undisrupted cells were pelleted at 500 g for 10 min at 4°C. The cavitate was fractionated as described by Eklund and Gabig, *J. Biol. Chem.*, *265*: 8426-8430 (1990). Briefly, it was layered over 40% sucrose in relaxation buffer containing 2 mM EGTA, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine, and centrifuged at 104,000 x *g* (at r_{av}) for 45 min at 4°C in a rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA). The top layer (FX_1), the 40% sucrose layer (FX_2), and the granule pellet (FX_3) were collected and assayed for lactate dehydrogenase as a cytoplasmic marker, alkaline phosphatase as a

protein/ml resin) hooked in series to a P selectin-Affigel 15™ column (0.6 x 13 cm, 2 mg protein/ml resin). The columns were equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, 0.01% Lubrol™ PX. After the samples were applied the columns were washed with 100 column volumes of equilibration buffer, and eluted with equilibration buffer containing 5 mM EDTA. Yields were estimated by protein assays with the Micro BCA protein assay kit using BSA as a standard.

Metabolic Radiolabeling of HL-60 Cells and Isolation of [^{3}H]glucosamine-labeled P-selectin Ligand

HL-60 cells (1-2 x 10^6 cells/ml) in 100-mm tissue culture dishes were labeled for 48 h with 50 μCi/ml [6-^{3}H]glucosamine at 37°C in RPMI-1640 containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. At the end of the labeling periods the cells were washed three times by centrifugation and resuspension in ice-cold PBS. The cell pellet was solubilized with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 1% Triton X-100™, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 8 μg/ml pepstatin, 2 mM PMSF, 10 mM benzamidine, and 0.5 mM dichloroisocoumarin. The solubilized cells were allowed to sit on ice for 1-2 h and then sonicated for 20 min at 4°C in a water bath sonicator. The cell extract was centrifuged for 5 min at 16,000 x *g* and the supernatant was applied to a P-selectin-Affigel 15™ column (0.25 x 13 cm, 2 mg protein/ml resin) equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% Triton X-100. The column was washed with 10-20 column volumes of equilibration buffer and bound material was eluted with equilibration buffer containing 10 mM EDTA. Fractions (1 ml) were collected and monitored for radioactivity by liquid scintillation counting. Samples of the run-through fractions and the bound, EDTA-eluted fractions were analyzed by SDS-PAGE under reducing conditions and fluorography. A single major glycoprotein of approximately 120 kD was isolated. In non-reducing SDS-PAGE, this glycoprotein species had a mobility corresponding to approximately 250 kD. Treatment of the 120 kD glycoprotein with neuraminidase abolished binding to P-selectin and caused an unusual decrease in electrophoretic mobility of the

acetylgalactosaminidase (70 mU/ml at pH 6.5) in the presence of 5 mM PMSF and 5 mM 1,10 phenanthroline.

Affinity purified [^{3}H]glucosamine-labeled P-selectin ligand was incubated for 24 h in 25 mM sodium acetate, pH 5.5 at 37°C under a toluene atmosphere in the presence or absence of 1 U/ml of *A. ureafaciens* sialidase for 18 h. For PNGaseF digestion of metabolically labeled ligand, samples were denatured by boiling in 0.25% SDS, 25 mM β-mercaptoethanol for 5 min, and NP-40 was added in eight-fold excess (wt/wt) over SDS. The samples were incubated for 24 h with PNGaseF (3.3 U/ml) in a toluene atmosphere. The samples were then precipitated with TCA and subjected to SDS-PAGE and fluorography as described above.

Flow Cytometry

Human neutrophils, isolated as described by Hamburger and McEver, (1990), were suspended (10^6/ml) in HBSS containing 1% FCS and 0.1% sodium azide (HBSS/FCS/Az). 1 ml of neutrophil suspension was underlaid with 100 µl FCS and centrifuged at 500 g for 5 min. The neutrophil pellet was resuspended in 50 µl of purified P selectin (10 µl/ml, in HBSS/FCS/Az), and then incubated sequentially with 50 µl of biotin-conjugated S12 (10 µg/ml, in HBSS/FCS/Az) and 20 µl of phycoerythrin-streptavidin (neat). In certain experiments, the neutrophils were preincubated for 10-15 min with antisera or antibodies before the addition of P-selectin. Between each step the cells were diluted with one ml of HBSS/FCS/Az, underlaid with 100 µl FCS, and centrifuged at 500 g for 5 min. All steps were performed at 4°C. After the last wash, the cells were fixed with 1 ml of 1% paraformaldehyde in HBSS and analyzed in a FACScan™ flow cytometer Becton Dickinson & Co., Mountain View, CA) formatted for two color analysis as

antipain, 1 mM benzamidine, and the extract applied to a wheat germ agglutinin (WGA) affinity column (1.5 x 20 cm, 7.6 mg lectin/ml resin, Vector Laboratories), equilibrated at room temperature with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 1% Triton X-100™. The column was washed with two column volumes of equilibration buffer, followed by four column volumes of 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 0.2% Brij-58. The column was then eluted with the above buffer containing 500 mM N-acetylglucosamine. Protein-containing fractions were pooled and subjected to an additional affinity chromatographic step using a P-selectin-Emphaze™ column. The pooled fractions were made 8 mM in $CaCl_2$ and applied to the P-selectin-Emphaze™ column (0.6 x 14 cm, 7.5 mg protein/ml resin) equilibrated with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% sodium azide, 0.02% Brij-58. Recombinant truncated P-selectin (tPS, see Ushiyama et al., *J. Biol. Chem.*, *268*: 15229-15237 (1993)) was coupled to 3M Emphaze™ Biosupport Media (Pierce Chemical Co.) according to the manufacturer's instruction in 0.1 M MOPS, pH 7.5, 0.8 M Na citrate. The column was washed with 10 column volumes of equilibration buffer, and eluted with equilibration buffer containing 5 mM EDTA. The tPS column was loaded at a flow rate of 0.1 ml/min, washed at 1 ml/min and eluted at 0.1 ml/min.

P-selectin ligand-containing fractions eluted from the tPS-Emphaze™ column were pooled and loaded onto an anion exchange column, Mono Q PC 1.6/5 column equilibrated with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 0.02% Brij-58 using a SMART™ Micro Separation System (Pharmacia/LKB). The sample was loaded at 0.1 ml/min, washed with several column volumes of

methanol. The cartridge was centrifuged at 4500 x *g* for one hour in a Fisher Model 59A Microfuge equipped with a swing-out rotor. After the sample was applied, the PVDF membrane was washed twice with 400 µl of HPLC grade H_2O. The PVDF membrane was removed using a Prospin™ Membrane Removal Punch (Applied Biosystems) and washed ten times with 1 ml of HPLC grade H_2O. After the last wash was removed, the PVDF membrane was frozen on dry ice.

The sample (designated gp120) was shipped on dry ice to Harvard Microchem (16 Divinity Avenue, Cambridge, MA 02138) for N-terminal sequencing and *in situ* trypsin digestion and HPLC separation of peptides.

RESULTS

Identification of a P-selectin Ligand

To identify proteins from myeloid cells which bind P-selectin, neutrophil and HL-60 cell membrane extracts were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to Immobilon™ membranes, and probed with [^{125}I]P-selectin. When samples were analyzed without reduction, P-selectin bound preferentially to a glycoprotein species with an approximately 250,000 M_r from both neutrophil and HL-60 cell membranes as determined by SDS-PAGE. Cell membrane extracts (80 µg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing or reducing conditions, transferred to Immobilon™ membranes, and probed with [^{125}I]P-selectin. Under nonreducing conditions P-selectin also bound to proteins at the stacking gel interface and to a minor species with an approximately 160,000 M_r. When samples were analyzed after reduction, P-selectin preferentially bound to a glycoprotein with an approximately 120,000 M_r. Minor bands were observed at approximately 250,000 and approximately 90,000 M_r.

selectin. The binding of [^{125}I]P-selectin to the dye front and to the 90,000 D protein was not blocked by EDTA or G1, suggesting that these interactions were nonspecific or used a specific Ca^{2+}-independent recognition mechanism.

Purification of P-selectin Ligand from Neutrophils

Neutrophils were disrupted and the membrane fraction (FX_2) isolated by fractionation of the cavitate as described in Materials and Methods. The membrane fraction constituted approximately 5% to 7% (n>10) of the protein in the cavitate. This fractionation depleted both cytosolic proteins and azurophilic granules as shown by Table I. Proteins binding P-selectin were not detected in the cytosolic fraction (FX_1) with the blotting assay. The final membrane pellet was solubilized with nonionic detergent and applied to a WGA column which bound 4-5% of the protein in the membrane extract. P-selectin blotting assays of reduced proteins demonstrated that both the major 120,000 D and the minor 250,000 D ligands bound quantitatively to WGA. However, the 90,000 D band and the band at the dye front observed in the membrane extract were not bound by WGA. After extensive dialysis, the WGA eluate was applied to an Affigel 15™ precolumn in series with a P-selectin affinity column. Approximately 2% of the protein in the WGA eluate bound to the P-selectin column and could be eluted with EDTA. Both the 250,000 D and the 120,000 D ligands bound quantitatively to the P-selectin column. Quantitative analysis of the protein recovered from the P-selectin eluate indicated that the ligand(s) formed less than 0.01% of the total protein in the neutrophil cavitate. Elution of bound proteins from the P-selectin column with EDTA demonstrated

selectin and were recognized by anti-P-selectin IgG. The P-selectin ligand identified in the blotting assay was not detected by silver staining and migrated differently than P-selectin under both reducing and nonreducing conditions. When the P-selectin eluate was electrophoresed without reduction, P-selectin did not bind to proteins at the stacking gel interface. Therefore, the P-selectin binding proteins at the stacking gel interface, observed in extracts of neutrophil membranes, were probably an artifact due to the relatively high amount of protein loaded on the gel.

Characterization of the P-selectin Ligand from Neutrophils

The ligand on intact target cells requires sialic acids to interact with P-selectin. To determine whether the ligand detected by blotting of neutrophil membranes contained sialic acids that were essential for recognition by P-selectin, neutrophil membrane glycoproteins which bound to WGA were treated with sialidase (200 mU/ml) for varying times before SDS-PAGE under reducing conditions and then analyzed for their ability to bind P-selectin. Neutrophil WGA eluate (50 μg) was either sham-treated or digested with 200 mU/ml of sialidase or with 20 U/ml of PNGaseF for 16 h, then electrophoresed on 7.5% SDS polyacrylamide gels under reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin. Sialidase digestion for 30 min increased the apparent molecular weight of the major 120,000 D ligand, a shift characteristic of heavily sialylated glycoproteins. Longer sialidase digestion did not further alter the electrophoretic mobility of the ligand but did abolish its ability

loss of P-selectin binding, the digestions were repeated in the presence of a competitive sialidase inhibitor, Neu2en5Ac. Under these conditions endo-α-*N*-acetylgalactosaminidase digestion had no effect on [^{125}I]P-selectin binding to the ligand or the apparent molecular weight of the ligand. Because the ligand requires sialic acid to interact with P-selectin, the blotting assay could not be used to assess the role of O-linked glycans in recognition by P-selectin.

Isolation of a P-selectin Ligand from Metabolically Labeled HL-60 Cells

P-selectin blotting of denatured membrane proteins from myeloid cells may not detect molecules whose ability to bind P-selectin is dependent on secondary and/or tertiary structure. As an independent approach to identify ligands for P-selectin, HL-60 cells were metabolically labeled with [^{3}H]glucosamine, solubilized with nonionic detergent, and applied to a P-selectin affinity column. After extensive washing, bound material was eluted with EDTA and analyzed by SDS-PAGE followed by fluorography. Samples were electrophoresed on 10% SDS polyacrylamide gels under both nonreducing and reducing conditions and analyzed by fluorography. Other samples were either sham treated or digested with 1 U/ml of sialidase for 24 h or with 3.3 U/ml of PNGaseF for 24 h, and then electrophoresed on 10% SDS polyacrylamide gels under reducing conditions and analyzed by fluorography.

A single metabolically labeled species was eluted, which co-migrated under both nonreducing and reducing conditions with the major species detected in neutrophil and HL-60 cell membranes by blotting with [^{125}I]P-selectin. Only 0.15-0.5% of the total [^{3}H]glucosamine-labeled HL-60

proteins under nonreducing conditions were distinct from that of the P-selectin ligand. In contrast to the P-selectin ligand, the electrophoretic mobilities of lamp-1 and lamp-2 are not affected by sialidase treatment. Although lamp-1 and lamp-2 from myeloid cells are rich in lactosaminoglycans sensitive to endo-ß-galactosidase, treatment of intact neutrophils with the enzyme did not affect binding of [^{125}I]P-selectin. Pretreatment of crude neutrophil membrane extracts or WGA column eluate with endo ß-galactosidase (200 mU/ml, 1-2 h, 37°C) also did not affect the apparent molecular weight of the ligand or its ability to bind [^{125}I]P-selectin. These data argue that lamp-1 and lamp-2 are not ligands for P-selectin even though they carry many sialyl Le^x structures.

The third molecule whose apparent molecular weight is similar to the 120,000 D P-selectin ligand is CD43 (leukosialin, sialophorin), a heavily sialylated membrane protein present on platelets and all leukocytes. It carries numerous O-linked sugar chains and is differentially glycosylated by cells of various hematopoietic lineages. Like the P-selectin ligand, treatment of leukosialin with sialidase increases its apparent molecular weight. However, in contrast to the P-selectin ligand, the electrophoretic mobility of leukosialin was unaffected by reduction. Monospecific polyclonal anti-human leukosialin antisera (1:5 dilution) did not inhibit P-selectin binding to neutrophils as assessed by flow cytometry. Furthermore, immunodepletion of leukosialin from neutrophil membrane extracts did not deplete P-selectin ligand as assessed by the blotting assay. Finally, leukosialin purified from HL-60 cells did not bind P-selectin. Neutrophil WGA eluate (50 µg) and leukosialin purified from

contribute to the binding of fluid-phase P-selectin to intact neutrophils or to immobilized proteins from neutrophil membrane extracts.

The following additional determinations and observations have been made relating to the glycoprotein ligand for P-selectin.

Amino Acid Sequences of Peptides of the P-selectin Ligand from Human Neutrophils

Amino acid sequence data on tryptic peptides derived from the sample sent to Harvard Microchem revealed two peptides that did not match any known sequence in the Brookhaven Protein Data Bank, SWISS-PROT or PIR protein sequence databases, or the translated GENBANK database. These two peptides HMYPVR (Sequence I.D. No. 1) and PGLTPEP (Sequence I.D. No. 2) correspond to amino acids 340-345 and amino acids 380-386 respectively of a cDNA cloned from an HL-60 cDNA library, reported by T. M. Veldmann of Genetics Institute at the meeting on "Cell Adhesion: Regulation and Clinical Prospects" in Amsterdam on October 15, 1993. Based on limited functional and structural data presented by Dr. Veldmann, it appears likely that this cDNA represents the P-selectin ligand.

Demonstration that the 120 kD P-selectin Ligand from Human Neutrophils Contains N-linked Oligosaccharides That Are Not Required for P-selectin Binding

Studies described above demonstrated that the P-selectin ligand contains a limited number of N-linked glycan chains and that enzymatic removal of these chains with PNGaseF did not affect the ability of the ligand to bind [^{125}I]P-selectin using the P-selectin blotting assay. To address this question using an independent and more quantitative approach [^{125}I]P-selectin ligand was digested with

anhydride according to standard procedures. The remaining radioactivity in the glycopeptides was composed of N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) in the approximate ratio of 1:2, respectively. This indicates that the overall composition of amino sugars in the 120 kD P-selectin ligand is approximately 25% sialic acid, 50% GlcNAc and 25% GalNAc. The P-selectin ligand purified from human neutrophils was analyzed by Dionex HPAE chromatography following neuraminidase treatment and all sialic acid was recovered as N-acetylneuraminic acid (see also, Norgard et al., 1993). One microgram of the purified human neutrophil ligand of 120 kD was hydrolyzed with 2 N trifluoroacetic acid according to standard procedures, and the released monosaccharides (except for sialic acid which is destroyed by this treatment) were analyzed by high performance anion exchange chromatography on a PA-1 Dionex column and detected by pulsed amperometric detection. The results indicated that the sample contained 233 picomoles of fucose, 70 picomoles of $GalNH_2$ (recorded as GalNAc), and approximately 200 picomoles of $GlcNH_2$ (recorded as GlcNAc). On a molar basis, assuming the molecular weight of the ligand to be in the range of 100,000 for sake of argument, then 1 microgram is approximately 10 picomoles of glycoprotein. This suggests that each mole of ligand contains approximately 23 moles of fucose, 7 moles of GalNAc and 20 moles of GlcNAc.

The P-selectin ligand from HL-60 cells was purified from cells grown in media containing either [2-^{3}H]mannose or [^{14}C]fucose. These precursors allow specific radiolabeling of mannose and fucose residues, respectively. Both radioactive mannose and fucose were recovered in the purified P-selectin ligand, confirming that it,

In a second approach, [^{3}H]glucosamine-labeled P-selectin ligand was purified from HL-60 cells and subjected to treatment with mild base (0.05 M NaOH) in the presence of sodium borohydride (1 M) for 16 h at 45°C. This condition effects the β-elimination reaction and release of the intact O-linked oligosaccharides with conversion of the linking GalNAc to N-acetylgalactosaminitol. This material was analyzed by gel filtration on a column of BioGel P-10™, which separates oligosaccharides primarily on the basis of their size. Such techniques for the structural analysis of a mucin-like glycoprotein from metabolically-radiolabeled cells have previously been used in the analysis of the LDL-receptor and more recently on studies of the human transferrin receptor (Cummings, R.D., Kornfeld, S., Schneider, W.J., Hobgood, K.K., Tolleshaug, J., Brown, M.S., and Goldstein, J.L., *J. Biol. Chem.*, *258*: 15261-15273 (1983); Do, S.-I., and Cummings, R.D., *Glycobiology*, *2*: 345-353 (1992), incorporated herein by reference. Before the β-elimination reaction all of the radiolabeled P-selectin ligand eluted in the void volume of the column, as expected, indicating that the intact material is large in size. After the β-elimination reaction most of the radioactivity was included in the column eluting in a size range of 1,000 to 1,500 daltons. This is a typical elution position for sialylated O-linked oligosaccharides. The unreleased N-linked oligosaccharides (now contained on a base-hydrolyzed peptide) elute in a peak near the void volume. The β-elimination reaction was also performed on the 120 kD glycoprotein ligand derived from human neutrophils. In that case the ligand was post-radiolabeled on its sialic acid by periodate oxidation followed by reduction with NaB_3H_4. The β-eliminated material from the

Evidence that the 120 kD P-selectin Ligand Contains SLe^x Antigen

Three alternative approaches were used to show that the 120 kD P-selectin ligand contains sLe^x. In the first approach, the ^3H-glucosamine-labeled P-selectin ligand from HL-60 cells was reapplied to a column of P-selectin-Affigel™. When this chromatography was done in the presence of antibody to the SLe^x antigen (CSLEX1 monoclonal antibody, see Fukushima, et al., *Cancer Res.*, *44*: 5279-5285 (1984), purchased from Dr. Paul Teraski, University of California, Los Angeles) binding was more than 90% reduced. In contrast, when a control experiment was done in which the rechromatography occurred in the presence of antibody to the Le^x antigen, which lacks sialic acid, there was little if any effect.

In an alternative approach, the 120 kD glycoprotein ligand from human neutrophils was purified by affinity chromatography on a column of immobilized soluble truncated P-selectin (tPS) (Ushiyama et. al. *J. Biol. Chem.*, *268*: 15229-15237 (1993)). The material was analyzed by SDS-PAGE in reducing conditions, transferred to Immobilon™ membrane, and probed for its reactivity with radioiodinated P-selectin and the monoclonal antibody CSLEX-1, which reacts with SLe^x. The 120 kD glycoprotein eluted from the P-selectin affinity column reacts with ^{125}I-P-selectin. The same material reacts with antibody to SLe^x, using the technique of Western blotting. These results demonstrate that the neutrophil-derived 120 kD glycoprotein ligand for P-selectin contains SLe^x.

As another approach to this problem, the ^{125}I-labeled form of the neutrophil-derived 120 kD glycoprotein ligand for P-selectin, prepared as described above, was analyzed for its ability to

endo-β-galactosidase because of the terminal sialic acids. This explains why neither the binding of the radioiodinated neutrophil-derived P-selectin ligand to immobilized P-selectin nor to immobilized CSLEX-1 antibody is abolished by treatment with endo-β-galactosidase.

Demonstration that the 120 kD P-selectin Ligand from HL-60 cells is Sulfated

HL-60 cells were metabolically-radiolabeled with $Na^{35}SO_4$ to examine whether the 120 kD ligand for P-selectin is sulfated. Approximately 2 x 10^6 cells/ml were grown in media containing 0.15 mCi/ml of $Na^{35}SO_4$ for 48 h in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The P-selectin glycoprotein ligand was then purified as described above. Fractions (1 ml) from the P-selectin-immobilized affinity column were collected and radioactivity monitored by liquid scintillation counting. The metabolically-radiolabeled glycoprotein eluted from the P-selectin column was precipitated by addition of ice-cold trichloroacetic acid (10% final). The pellets were washed with 1 ml of cold acetone, 0.2% HCl, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE in 7.5% acrylamide. The gel was processed for fluorography with EN^{3}HANCE™ according to the manufacturer's instructions. The dried gel was exposed to Fuji RX film at -80°C for 7 days. The results indicated that the 120 kD glycoprotein bound by P-selectin is radiolabeled by $^{35}SO_4$.

The differential mobility of the major ligand during SDS-PAGE in the presence and absence of reducing agents indicates that the native ligand is a disulfide-linked homodimer. A homodimeric ligand with two equivalent binding sites might enhance the

extracts was not detectable. Furthermore, the binding of P-selectin to intact neutrophils is unaltered by antibodies to L-selectin or by neutrophil activation that causes shedding of L-selectin from the cell surface. Although it is conceivable that L-selectin has weak affinity for P-selectin, the significance of this potential interaction remains to be established.

A recombinant P-selectin IgG chimera was shown to bind to myeloid cells and to a sulfatide, Gal(3-SO_4) ß1-Ceramide by Aruffo et al., *Cell*, *67*: 35-44 (1991). Sulfatide also inhibited interaction of the chimera with monocytoid U937 cells, as reported by Aruffo et al., (1991). It was not demonstrated whether binding of the P-selectin chimera to the cells or to sulfatide was Ca^{2+} dependent, a fundamental characteristic of selectin-dependent cellular interactions. Protease digestion of intact cells should increase the accessibility of P-selectin to potential glycolipid ligands such as sulfatides. However, protease treatment abolishes binding of P-selectin to neutrophils and HL-60 cells as well as adhesion of neutrophils to immobilized P-selectin. In addition, although erythrocytes and platelets express sulfatides, they do not specifically interact with P-selectin. Thus, it seems unlikely that sulfatides are the principal mediators of adhesion of myeloid cells to P-selectin. It remains to be determined whether sulfatides inhibit binding of P-selectin to myeloid cells by specific competition with a glycoprotein ligand or by indirect effects. Because the P-selectin ligand described herein is sulfated, it may contain structural features that are mimicked by sulfatides.

Previous studies by Zhou et al., (1991); and Polley et al., (1991) have shown that P-selectin

EDTA. The glycoprotein ligand was greatly enriched in the EDTA eluate from the P-selectin column, as assessed by the intensity of the band identified by [^{125}I]P-selectin blotting. As noted in WO 92/01718, however, the ligand stained poorly with silver, consistent with its being an unusually heavily glycosylated protein. In the initial purifications, the only contaminating protein present noted by silver staining of the gel was a small amount of P-selectin itself which had been leached from the affinity column. Using a new P-selectin affinity column and more extensive washing procedures documented in the methods, the ligand has now been isolated free from contaminants. This conclusion is based on observation that there are no silver staining bands present but the ligand is clearly identified by its ability to interact with [^{125}I]P-selectin in the blotting assay.

As described in WO 92/01718, partial removal of sialic acids with sialidase slowed the mobility of the ligand, a feature characteristic of heavily sialylated glycoproteins. Extensive sialidase digestion abolished recognition of the ligand by P-selectin. It has now been demonstrated that the ligand contains both N- and O-linked oligosaccharides. Further, the material was shown to be pure as assessed by SDS-PAGE and autoradiography following radioiodination.

A form of the ligand in which the carbohydrate components are radiolabeled has also been purified by P-selectin affinity chromatography, as described above. SDS-PAGE analysis of the P-selectin column eluate, followed by fluorography, indicates that the only labeled protein has an Mr of 250,000 under nonreducing conditions and 120,000 under reducing conditions. The radiolabeled ligand has the same shifts in electrophoretic mobility following

Preparation of Diagnostic and Therapeutic Agents Derived from the Protein or Carbohydrate Components of the Glycoprotein Ligand for P-selectin.

The glycoprotein ligand for P-selectin described above has a variety of applications as diagnostic reagents and, potentially, in the treatment of numerous inflammatory and thrombotic disorders.

Diagnostic Reagents

Antibodies to the ligand, fragments thereof, or its carbohydrate or polypeptide components, can be prepared by methods known in the art (e.g., Harlow, E. and Lane, D., in Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988). Such antibodies can be used for the detection of human disorders in which P-selectin ligands might be defective. Such disorders would most likely be seen in patients with increased susceptibility to infections in which leukocytes might not be able to bind to activated platelets or endothelium. Cells to be tested, usually leukocytes, are collected by standard medically approved techniques and screened. Detection systems include ELISA procedures, binding of radiolabeled antibody to immobilized activated cells, flow cytometry, immunoperoxidase or immunogold analysis, or other methods known to those skilled in the arts.

Antibodies directed specifically to protein or carbohydrate components of the ligand can be used to distinguish defects in expression of the core protein or in glycosyltransferases and/or modifying enzymes that construct the proper oligosaccharide chains on the protein. The antibodies can also be used to screen cells and tissues other than leukocytes for expression of the protein or

A similar approach can be used to determine qualitative or quantitative disorders of P-selectin itself. The glycoprotein ligand, carbohydrates, or appropriate derivatives thereof, is labeled and tested for its ability to bind to P-selectin on activated platelets from patients with disorders in which P-selectin might be defective.

The ligand, or components thereof, can also be used in assays of P-selectin binding to screen for compounds that block interactions of P-selectin with the ligand.

Clinical Applications

Since P-selectin has several functions related to leukocyte adherence, inflammation, tumor metastases, and coagulation, clinically, compounds which interfere with binding of P-selectin and/or the other selectins, including E-selectin and L-selectin, such as the carbohydrates, can be used to modulate these responses. These compounds include the P-selectin ligand, antibodies to the ligand, and fragments thereof. For example, the glycoprotein ligand, or components thereof, particularly the carbohydrate moieties, can be used to inhibit leukocyte adhesion by competitively binding to P-selectin expressed on the surface of activated platelets or endothelial cells. Similarly, antibodies to the ligand can be used to block cell adhesion mediated by P-selectin by competitively binding to the P-selectin ligand on leukocytes or other cells. These therapies are useful in acute situations where effective, but transient, inhibition of leukocyte-mediated inflammation is desirable. In addition, treatment of chronic disorders may be attained by sustained administration of agents, for example, by subcutaneous or oral administration.

transplantation; and circulatory shock (in this case many organs might be damaged following restoration of blood flow).

Bacterial sepsis and disseminated intravascular coagulation often exist concurrently in critically ill patients. They are associated with generation of thrombin, cytokines, and other inflammatory mediators, activation of platelets and endothelium, and adherence of leukocytes and aggregation of platelets throughout the vascular system. Leukocyte-dependent organ damage is an important feature of these conditions.

Adult respiratory distress syndrome is a devastating pulmonary disorder occurring in patients with sepsis or following trauma, which is associated with widespread adherence and aggregation of leukocytes in the pulmonary circulation. This leads to extravasation of large amounts of plasma into the lungs and destruction of lung tissue, both mediated in large part by leukocyte products.

Two related pulmonary disorders that are often fatal are in immunosuppressed patients undergoing allogeneic bone marrow transplantation and in cancer patients suffering from complications that arise from generalized vascular leakage resulting from treatment with interleukin-2 treated LAK cells (lymphokine-activated lymphocytes). LAK cells are known to adhere to vascular walls and release products that are presumably toxic to endothelium. Although the mechanism by which LAK cells adhere to endothelium is not known, such cells could potentially release molecules that activate endothelium and then bind to endothelium by mechanisms similar to those operative in neutrophils.

interactions by binding competitively to P-selectin expressed on activated cells. In addition, carbohydrate components of the ligand, which play a key role in recognition by P-selectin, can be administered alone, as well as attached to all or a fragment of the polypeptide component of the ligand. Similarly, natural or synthetic analogs of the ligand or its fragments which bind to P-selectin can also be administered to a patient to block P-selectin dependent interactions. In addition, antibodies to the polypeptide and/or carbohydrate components of the ligand, or fragments thereof, can be administered. The antibodies are preferrably of human origin or modified to delete those portions most likely to cause an immunogenic reaction. The ligand, or fragments thereof, carbohydrate components of the ligand, and antibodies to the ligand molecule or its carbohydrate or polypeptide components, in an appropriate pharmaceutical carrier, are preferably administered intravenously where immediate relief is required. Other modes of administration include intramuscularly, intraperitoneally, subcutaneously, and orally. The carbohydrate component of the ligand may also be conjugated to a carrier molecule, or incorporated into a drug delivery device for more effective and prolonged delivery to a patient. The carbohydrate can also be modified chemically to increase its *in vivo* half-life.

The carbohydrate can be isolated from cells expressing the carbohydrate, either naturally or as a result of genetic engineering as described in the transfected COS cell examples, or, preferably, by synthetic means. These methods are known to those skilled in the art. In addition, a large number of glycosyltransferases have been cloned (J.C. Paulson and K.J. Colley, *J. Biol. Chem.*, *264*: 17615-17618

include lubricants, flavorings, binders, and other materials of the same nature. The carbohydrate, ligand, or fragments thereof, can also be administered locally at a wound or inflammatory site by topical application of a solution or cream.

Alternatively, a carbohydrate component of the ligand, the ligand, or fragments thereof, may also be administered in, on or as part of, liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A good review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the carbohydrate components of the P-selectin ligand, the ligand, or fragments thereof, can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patent No. 4,906,474, 4,925,673, and 3,625,214.

The carbohydrates should be active when administered parenterally in amounts above about 1 μg/kg of body weight. For treatment of most inflammatory disorders, the dosage range will be between 0.1 to 30 mg/kg of body weight. A dosage

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Board of Regents of the University of Oklahoma

(ii) TITLE OF INVENTION: Glycoprotein Ligand For P-Selectin and Methods of Use Thereof

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Patrea L. Pabst
(B) STREET: 1100 Peachtree Street, Suite 2800
(C) CITY: Atlanta
(D) STATE: Ga
(E) COUNTRY: USA
(F) ZIP: 30309-4530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/650,484
(B) FILING DATE: 05-FEB-1991

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/554,199
(B) FILING DATE: 17-JUL-1990

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/320,408
(B) FILING DATE: 08-MAR-1989

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: OMRF110CIP(4)

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (404)-815-6508
(B) TELEFAX: (404-815-6555

We claim:

1. A ligand for P-selectin comprising a fucosylated sialylated glycoprotein containing glucosamine and galactosamine in the approximate ratio of 2:1, N-linked oligosaccharides, O-linked oligosaccharides or Ser/Thr-linked oligosaccharides containing the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-acetyllactosamine sequences, wherein the ligand has an apparent relative molecular weight of 120,000 as assessed by SDS-PAGE under reducing conditions, an apparent molecular weight of 250,000 as assessed by SDS-PAGE under non-reducing conditions.

2. The ligand of claim 1 comprises the amino acid sequences HMYPVR (Sequence I.D. No. 1) and PGLTPEP (Sequence I.D. No. 2).

3. The ligand of claim 1 wherein the ligand is isolated from myeloid cell membranes of human neutrophils:

solubilizing the membranes of the myeloid cells to form a membrane extract, binding the membrane extract to an affinity chromatography column containing immobilized P-selectin, eluting the ligand from the affinity column with a calcium chelating agent loading the pooled ligand.

4. The ligand of claim 3 wherein the eluted ligand is further purified by chromatography on a high performance liquid chromatography column.

5. The ligand of claim 1 in the form of a dimer of the 120,000 molecular weight glycoprotein having a relative molecular weight by SDS-PAGE of 250,000 under non-reducing conditions.

SDS-PAGE under reducing conditions and an apparent molecular weight of 250,000 as assessed by SDS-PAGE under non-reducing conditions.

12. The protein of claim 11 comprising the amino acid sequences HMYPVR (Sequence I.D. No. 1) and PGLTPEP (Sequence I.D. No. 2).

13. An antibody to a ligand of P-selectin comprising a fucosylated sialylated glycoprotein containing glucosamine and galactosamine in the approximate ratio of 2:1, N-linked oligosaccharides, O-linked oligosaccharides or Ser/Thr-linked oligosaccharides containing the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-acetyllactosamine sequences, wherein the ligand has an apparent relative molecular weight of 120,000 as assessed by SDS-PAGE under reducing conditions, an apparent molecular weight of 250,000 as assessed by SDS-PAGE under non-reducing conditions.

14. The antibody of claim 13 wherein the antibody is specifically directed against a carbohydrate-protein component of the ligand.

15. The antibody of claim 13 wherein the antibody is immunoreactive against a polypeptide component of the ligand.

16. A method for modulating an inflammatory or hemostatic response comprising administering in a pharmaceutically acceptable carrier an effective amount of an agent selected from the group consisting of:

oligosaccharides or Ser/Thr-linked oligosaccharides containing the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-acetyllactosamine sequences, wherein the ligand has an apparent relative molecular weight of 120,000 as assessed by SDS-PAGE under reducing conditions, and an apparent molecular weight of 250,000 as assessed by SDS-PAGE under non-reducing conditions, a fragment of the glycoprotein, a polypeptide component of the glycoprotein and a carbohydrate component of the glycoprotein

in combination with a compound to be tested for inhibiting binding of ligand to P-selectin, and

(b) determining if binding of P-selectin to the ligand is inhibited by the compound to be tested.

20. A method of isolating a nucleic acid having a sequence encoding a ligand for P-selectin comprising:

(a) screening a human cDNA or genomic library with a labeled probe, wherein the probe is selected from the group consisting of:

degenerate synthetic nucleic acid molecules encoding either HMYPVR or PFLTPEP and

antibodies directed against the ligand for P-selectin or its carbohydrate components or its polypeptide components, wherein the ligand comprises a fucosylated sialylated glycoprotein containing glucosamine and galactosamine in the approximate ratio of 2:1, N-linked oligosaccharides, O-linked oligosaccharides or Ser/Thr-linked oligosaccharides containing the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr, sialyl Lewis x antigen NeuAcα2-3Galß1-4[Fucα1-3]GlcNAcß-R, and poly-N-acetyllactosamine

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 93/11129

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/10 C07K15/14 A61K37/02 A61K39/395 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELL BIOLOGY vol. 118, no. 2 , July 1992 , NEW YORK, USA pages 445 - 456 K. MOORE ET AL. 'Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells.' see the whole document ---	1,3-11
X	WO,A,92 01718 (REGENTS OF THE BOARD OF THE UNIVERSITY OF OKLAHOMA) 6 February 1992 cited in the application see claims see examples 5,6,8 --- -/--	1,3-11, 13-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 1994	08. 04. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11129

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.